52

Guidance for Industry

ASSESSMENT OF THE EFFECTS OF ANTIMICROBIAL DRUG RESIDUES FROM FOOD OF ANIMAL ORIGIN ON THE HUMAN INTESTINAL FLORA

DRAFT GUIDANCE

This guidance document is being distributed for comment purposes only

This draft document discusses a recommended pathway approach for assessing the effects of antimicrobial drug residues in food on the human intestinal flora. When this document is finalized, it will supercede the current guidance #52, Guideline for Microbiological Testing of Antimicrobial Drug Residues in Food, that published in January 1996.

Comments and suggestions regarding this document should be sent to the Dockets Management Branch (HFA 305), Food and Drug Administration, 5600 Fishers Lane, Room 1061, Rockville, MD 20852. All comments should be identified with the Docket No. 93 D-0398.

Direct questions regarding this document to Haydée Fernández, Division of Human Food Safety (HFV-153), Center for Veterinary Medicine, Food and Drug Administration, 7500 Standish Place, Rockville, MD 20855, 301-827-6981.

Additional copies of this draft guidance document may be requested from the Communications Staff (HFV-12), Center for Veterinary Medicine, Food and Drug Administration, 7500 Standish Place, Rockville, MD 20855 and may be viewed on the Internet at http://www.fda.gov/cvm.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Veterinary Medicine
December 19, 2001

ASSESSMENT OF THE EFFECTS OF ANTIMICROBIAL DRUG RESIDUES FROM FOOD OF ANIMAL ORIGIN ON THE HUMAN INTESTINAL FLORA

This document represents the Agency's current thinking on the approach that should be used to assess the microbiological safety of antimicrobial drug residues in food of animal origin. It does not create or confer any rights for or on any person and does not operate to bind the FDA or the public. An alternate approach may be used as long as it satisfies the requirements of applicable statutes and regulations.

I. INTRODUCTION

The human intestinal microflora is a balanced ecosystem that is very important in maintaining an individual's health. Although this system is generally stable, clinical studies have shown that therapeutic doses of antimicrobials may change the balance (1, 2, 3). The type or extent of change in the system will depend on the spectrum of action of the antimicrobial drug, its dose, and the length of an individual's exposure to the drug. The lowest concentration of any antimicrobial drug that can affect the intestinal microflora is not clear. However, studies in *in vitro* (chemostat systems) and *in vivo* human flora-associated rodent (rodents implanted with human fecal flora) model systems and in human volunteers have shown that low levels of antimicrobial drugs are capable of altering different parameters of the intestinal microflora depending on the spectrum of action and concentration of drug (4, 5, 2, 3).

The main adverse effects of antimicrobial drugs on the human intestinal microflora are selection of resistant bacteria and disruption of the colonization resistance (or barrier effect) of the resident intestinal microflora. Colonization resistance or barrier effect is the "limiting action" of the normal flora on colonization of the bowel by exogenous or indigenous potentially pathogenic microorganisms (6). Other effects, such as alteration of the metabolic activity of the flora, may be important, also.

Regulators and sponsors of new animal drugs have an interest in establishing relevant and validated methods for determining the effects of microbiologically active animal drug residues on the human intestinal flora. Any such effects need to be assessed in the human food safety evaluation of such new animal drugs intended for use in food-producing animals. Among the *in vitro* and *in vivo* approaches currently used to study the effect of antimicrobial drugs on the human intestinal microflora are quantitative *in vitro* antimicrobial drug susceptibility testing, static batch cultures, semi-continuous and continuous flow culture systems, simulated gut models, human volunteers, conventional animals, gnotobiotic rodents, and human flora-associated rodents.

In the Federal Register of January 30, 1996 (61 FR 3043), the Center for Veterinary Medicine (CVM, the Center) published a Notice of Availability of Guidance Document No. 52 "Microbiological Testing of Antimicrobial Drug Residues in Food" (Guidance

No. 52). This document stated that the CVM considers antimicrobial activity as a valid endpoint for establishing tolerances for antimicrobial drugs. The guidance also stated that antimicrobial drug residues present in food of animal origin should not cause any adverse effects on the ecology of the human intestinal microflora of consumers. The guidance identified antimicrobial drugs that would be exempt from additional microbiological testing and those that would warrant testing. The reasons for exempting certain antimicrobial drugs from additional microbiological testing included "very low" residues present in the food, residues with limited antimicrobial activity, and antimicrobial drugs with no adverse effects on the human intestinal microflora at doses approved for the target species (7).

Guidance No. 52 stated that "very low" levels of antimicrobial drug residues present in food of animal origin do not disrupt the intestinal microflora or select for resistant microorganisms and, therefore, would be "safe" under Section 512 of the Federal Food, Drug, and Cosmetic Act (the Act). Based on the best information available at that time, the CVM believed that a maximum Acceptable Daily Intake (ADI) of 1.5 mg/person/day of microbiologically active antimicrobial drug residues present in the food qualified as "very low" residues and should not produce adverse effects on the intestinal microflora (7). After CVM established the maximum ADI of 1.5 mg/person/day in the 1996 version of Guidance 52, CVM staff publicly stated (e.g., at a workshop sponsored by FDA on September 20 and 21, 1999, in Rockville, Maryland) that this threshold would need to be re-evaluated when additional information was obtained on the adequacy of this number for different classes of antimicrobial drugs.

The guidance recommended that additional microbiological testing be performed for those antimicrobial drugs for which sponsors were seeking an ADI higher than 1.5 mg/person/day. The guidance document identified the following areas for which antimicrobial residues present a potential public health concern. These endpoints are: 1) changes in the metabolic activity of the intestinal microflora; 2) changes in antimicrobial susceptibility patterns of the intestinal microflora; 3) changes in the colonization resistance properties (barrier effect) of the microflora; and 4) changes in the numbers and relative proportions of different bacterial species. The guidance recommended that sponsors characterize the product, identify its microbiological activity, and monitor the appropriate microbiological endpoints in order to establish the antimicrobial no-observed effect level (NOEL). Because no validated model systems were available at that time, the CVM announced its intention to validate model systems to evaluate the effect of low levels of antimicrobial drugs on endpoints of potential public health concern. The guidance also stated that *in vitro* minimum inhibitory concentration (MIC) data should not be submitted to establish the microbiological NOEL because these data are not predictive of the concentrations of drug residues that elicit potential public health concern. Sponsors were encouraged to consult with the CVM to determine appropriate protocols before conducting studies (7).

In 1995, the CVM funded two extramural research contracts to study the dose-response effects of antimicrobial drugs on human intestinal microflora endpoints that could be of public health concern. A continuous flow one-chambered chemostat inoculated with

human intestinal microflora and a human flora-associated (HFA) mouse model were studied as possible model systems for studying dose-response effects of low doses of antimicrobial drugs. FDA would expect that any model system to be used for regulatory purposes would be reproducible. (8).

In a workshop sponsored by FDA on September 20 and 21, 1999, in Rockville, Maryland, information from the two FDA-funded research contracts was presented. Data on the effect of low doses of different classes of antimicrobial drugs on several microbiological endpoints of the human intestinal microflora were discussed. After reviewing and discussing the data, the CVM concluded that the threshold ADI established in the 1996 version of Guidance No. 52 is not appropriate for all classes of antimicrobial drugs. Different classes of antimicrobial drugs affect to different degrees the microbiological endpoints that could be of public health concern. Therefore, the CVM has decided to modify Guidance No.52 to recommend that sponsors use a "pathway approach" (described below) for addressing the human food safety of antimicrobial drug residues rather than the approach described in the 1996 version of the guidance. The scientific rationale for this decision is provided in the Appendix of this draft document.

This draft guidance may be further revised at a later date in accordance with recommendations from an international government/industry guidance development group, the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), concerning proper tests, model systems, and standard protocols for addressing endpoints of public health concern (9). VICH also needs to address how to calculate ADIs using NOELs obtained from microbiological testing models. However, the CVM believes that it is in the best interest of the regulated industry and public health to revise this guidance now instead of waiting until the VICH recommendations are completed. The pathway approach presented here represents a general approach for assessing the microbiological safety of antimicrobial drug residues in food. If further microbiological studies are warranted for determining the ADI for a new animal antimicrobial drug, the sponsor of that drug is encouraged to contact the Center to discuss the appropriate test systems and protocols for the studies.

II. **THE GUIDANCE** - PATHWAY APPROACH FOR ADDRESSING THE EFFECTS OF ANTIMICROBIAL DRUG RESIDUES FROM FOOD OF ANIMAL ORIGIN ON THE HUMAN INTESTINAL FLORA

In the September 20 and 21, 1999 workshop "Microbiological Safety of Antimicrobial Residues in Food", the Center discussed a proposed pathway for addressing the microbiological safety of antimicrobial drug residues in food (8). The conditions and rationale for addressing the microbiological safety of these residues are simplified in a chart at the end of this section.

The microbiological safety of antimicrobial drug residues in food is a major issue that should be addressed by the sponsor of a new animal drug. An assessment of the safety of antimicrobial drug residues in food should be part of the human food safety component of new animal drug applications for antimicrobial drugs. If these residues are determined to have no antimicrobial activity against representatives of the human intestinal flora (*E. coli*, and species of *Bacteroides, Bifidobacterium, Clostridium, Enterococcus, Eubacterium (Collinsella), Fusobacterium, Lactobacillus, Peptostreptococcus/Peptococcus*), an ADI should be calculated based on traditional toxicology studies. However, if the residues have antimicrobial activity, the sponsor should address the potential availability of these microbiologically active residues in the human colon. It should be assumed that the human colon would be exposed to all residues present in the edible tissues, unless the sponsor can demonstrate through reference to controlled experimentation in humans or animals (e.g., pharmacokinetic studies of the same or similar antimicrobial drug) that some or all of the residues have no potential to enter the colon.

If it is determined that microbiologically active residues can enter the colon, the sponsor should assess the potential of these residues to select for resistant bacteria, disrupt the protective barrier effect provided by the intestinal microflora, or otherwise alter the balance of intestinal microflora. The sponsor may demonstrate that the residues are metabolized rapidly to microbiologically inactive compounds or are rapidly bound to intestinal contents and rendered microbiologically unavailable in the human colon. Alternatively, if the antimicrobial residues are not metabolized or bound such that they are microbiologically inactive, the sponsor should perform studies using an *in vitro or* an *in vivo* model system to determine the endpoint(s) of human health concern. The sponsor may wish first to perform preliminary studies such as batch cultures with fecal suspensions or an *in vivo* preliminary study to determine which microbiological endpoint is supected to be altered by the drug. The sponsor may also choose to perform a definitive study using an *in vitro* or an *in vivo* model system to determine the endpoint(s) of human health concern. However, if information exists on the class of drug, or preliminary studies show which endpoint(s) would be the most sensitive, definitive studies using *in vitro* or *in vivo* model systems should be performed to determine the NOEL for the drug on the chosen endpoint(s).

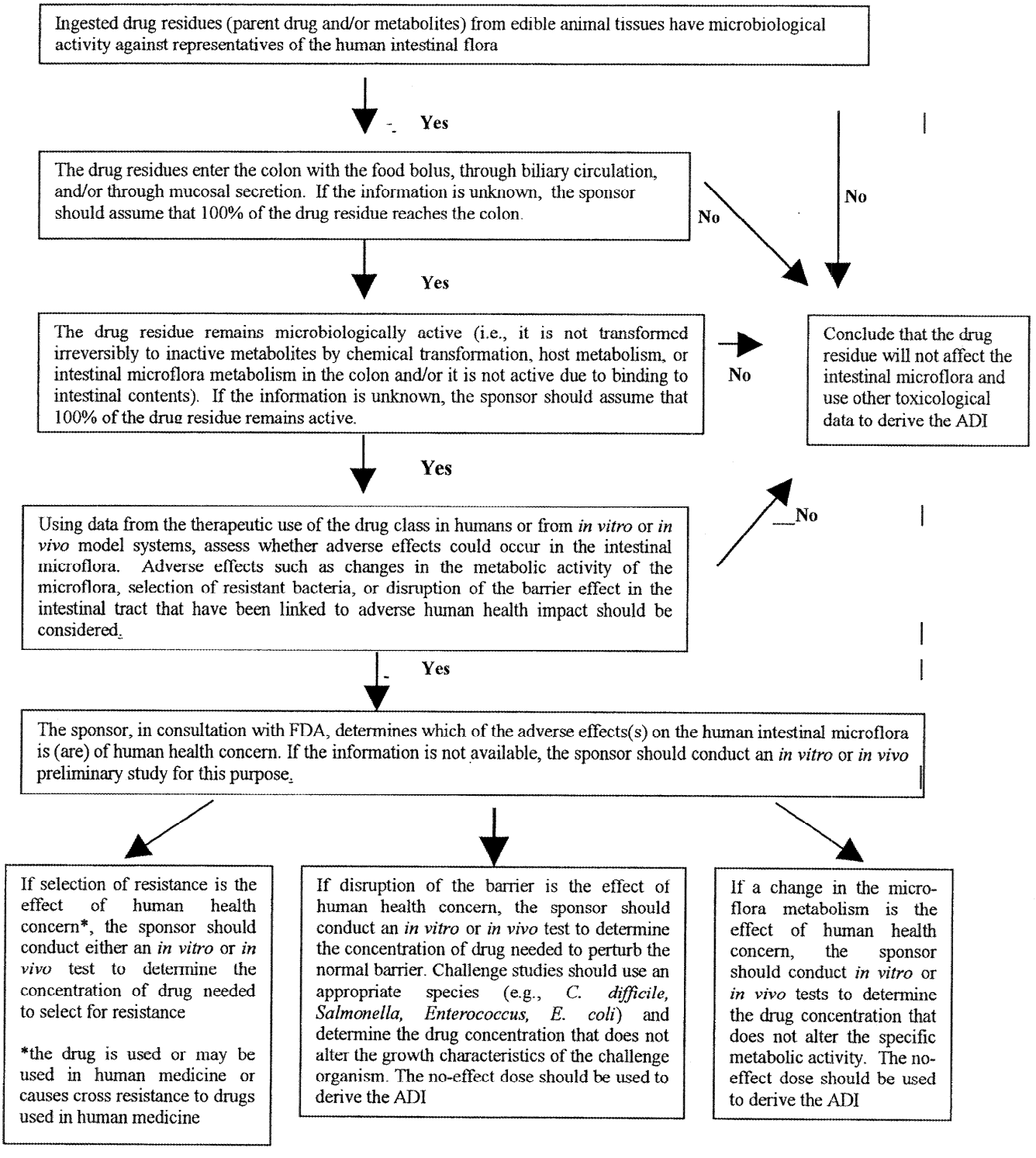
If the endpoint of concern is a change in the metabolic activity of the microflora, the sponsor should perform *in vitro* or *in vivo* studies to determine the NOEL for the endpoint. The NOEL should be used to calculate the ADI.

If disruption of the barrier effect is the endpoint of concern, either *in vitro* (e.g., continuous or semi-continuous culture systems) or *in vivo* model systems (e.g., human flora-associated rodent models) are preferable for determining a NOEL for this endpoint, as opposed to *in vitro* antimicrobial susceptibility testing to generate MIC data. This is because these models have the potential to better approximate the effects of microbial interactions and high bacterial densities. CVM does not encourage the development or use of MIC data for determining the NOEL for disruption of the barrier effect of the human intestinal microflora because quantitative *in vitro* determinations of antimicrobial

susceptibility do not reflect or account for factors such as bacterial population density, pH, intestinal growth conditions, bacterial metabolism, bacterial antagonism, or other factors of relevance to the human colonic microflora.

Finally, if the endpoint of concern is the selection of resistant bacterial strains, the sponsor should conduct *in vitro* or *in vivo* studies in model systems (see above) to determine a NOEL for this endpoint. Quantitative *in vitro* determinations of antimicrobial susceptibility, leading to the generation of MIC data that is coupled to the effects generated in the model system(s), should be an element of this analysis.

PATHWAY APPROACH FOR ADDRESSING THE EFFECTS OF ANTIMICROBIAL DRUG RESIDUES FROM FOOD OF ANIMAL ORIGIN ON THE HUMAN INTESTINAL FLORA



APPENDIX - SCIENTIFIC ISSUES AND CONCLUSIONS

A. Antimicrobials and the Human Intestinal Flora

The human intestinal microflora, an ecologically balanced system that plays an important role in maintaining and protecting the health of individuals, is generally very difficult to alter. For example, diet does not significantly alter the ecology of the human intestinal microflora. However, antimicrobial drugs may have a major effect on the ecology of the intestinal microflora (1, 4, 10).

Studies demonstrate that therapeutic oral doses of antimicrobials that are poorly or incompletely absorbed, excreted in the bile, or reach the intestinal lumen through circulation and excretion from the intestinal mucosa can potentially alter the intestinal microflora (8, 4, 5, 2). Some of these alterations may involve suppression of important bacteria and overgrowth of potentially pathogenic microorganisms that may cause systemic infections, especially in immunocompromised patients. A well-known example of overgrowth of potentially pathogenic bacteria is the infection caused by *Clostridium difficile*. This anaerobic bacterium, that may be indigenous or acquired in the hospital environment, may overgrow after being subjected to antibiotic treatment, disrupt the intestinal microflora, and produce toxins. *Clostridium difficile* toxins may damage the intestinal mucosa resulting in diarrhea that may range from mild to life-threatening pseudomembranous colitis (4).

Other effects of antimicrobial drugs include the selection of drug-resistant intestinal bacteria, the decrease of the colonization resistance properties of the flora (or barrier effect), and alteration of the metabolic activity of the intestinal bacteria (e.g., metabolism of neutral steroids and bile acids, conversion of bilirubin to urobilinogen, metabolism of drugs and other substances, synthesis of vitamins) (4, 2, 11, 3).

Different classes of antimicrobials may alter the intestinal microflora differently. For example, most quinolone drugs suppress or sometimes eliminate Gram-negative bacteria (especially *Enterobacteriaceae*), partially affect some Gram-positive aerobic cocci, and have very little effect on anaerobic bacteria. These alterations are concentration- and drug-dependent (2). Volunteers treated orally with 500 mg of ciprofloxacin every 12 hours for 7 days showed complete elimination of the coliform bacteria and a decrease in the number of streptococci and staphylococci by day 7; however, these counts returned to normal by day 14 (7 days after cessation of treatment). The anaerobic bacteria and yeasts were not affected and the flora returned to normal after cessation of ciprofloxacin treatment (12). Longer treatments with ciprofloxacin (500 mg given twice a day for 42 days to patients with leukemia) have shown to select for ciprofloxacin-resistant *Pseudomonas* and *Acinetobacter* (13). Ciprofloxacin-resistant *Escherichia coli* has emerged in cirrhotic patients treated with 1000 mg of ciprofloxacin once a week for 12 weeks. Similar effects are seen with other quinolones (14). Sitafloxacin, a broad-spectrum fluoroquinolone with activity against aerobic and anaerobic bacteria reduced the number of *Bacteroides* strains and other anaerobes; drastically reduced the number of *Enterobacteriaceae*, lactobacilli and bacilli; decreased the enterococci population in half of the volunteers; and increased the proportion of resistant *Bacteroides* strains to 6.25

μg/ml of drug in most patients treated orally with 100 mg of the drug three times a day for one week. Signs of recovery of the microflora were seen on day 14 after cessation of treatment, but the recovery was still incomplete (e.g., the proportion of resistant *Bacteroides* increased during the treatment period but decreased after the treatment) (15).

Other classes of antibiotics such as beta-lactams, cephalosporins, tetracyclines, lincosamides and macrolides, aminoglycosides, etc. may also produce effects on the intestinal microflora at therapeutic doses. Broad-spectrum penicillins at therapeutic doses have been shown to suppress the growth of aerobic Gram-negative bacteria and anaerobic microflora, and promote the overgrowth of aerobic Gram-positive bacteria. Also, most cephalosporins induce overgrowth or new colonization of resistant microorganisms during drug administration (16). Third generation cephalosporins have shown good activity against Gram-negative aerobic and anaerobic rods and imipenem, a broad-spectrum beta-lactam antibiotic of the carbapenem class has shown activity against the aerobic and anaerobic flora (17). Therapeutic doses of clindamycin may produce profound changes in the flora such as proliferation of resistant enterococci, decrease in the number of anaerobic cocci and rods, overgrowth of *Clostridium difficile*, and decrease in the number of *Escherichia coli* (5, 17, 18). Erythromycin, at therapeutic doses, has been shown to produce a drastic reduction in the number of enterococci and streptococci. This drug has also been shown to allow colonization of the colon by anaerobic bacteria and yeasts (5). Tetracyclines, e.g.,., doxycycline at therapeutic doses, have also been shown to increase the number of resistant anaerobic bacteria (17).

B. Residue Levels of Antimicrobial Drugs and Their Effect on the Intestinal Flora

The effect of antibiotic residues in food on the intestinal microflora of the consumer has been a concern for many years. However, the residue dose of antimicrobial drugs that adversely disturb the intestinal microflora has not been defined. Some research has been performed for evaluating the effect of residue levels of antibiotics on different endpoints of the human intestinal microflora. Oxytetracycline at doses of 10-50 mg/day has been shown to increase the excretion of resistant coliforms in some volunteers (19, 20); and even 2 mg/day given for 7 days produced a significant increase in the proportion of resistant *Enterobacteriaceae* in 6 volunteers (19, 21). Ampicillin given to 5 volunteers for 21 days at a dose of 1.5 mg/day produced a significant increase in resistant *E. coli* in two individuals. However, some authors have concluded that this trial lacked statistical power because there were not enough volunteers in the group (19, 22).

In vitro and *in vivo* model systems have also been used to study effects of antibiotic residues on human intestinal microflora. Milk safe residue levels of ampicillin, oxytetracycline, dihydrostreptomycin, neomycin, sulfamethazine, and erythromycin, as determined by FDA, showed a strong potential for selecting antibiotic-resistant *Staphylococcus aureus* (based on MIC determinations) when the microorganism was exposed for 14 days to each drug or to combinations of three drugs (23). Sub-inhibitory concentrations of streptomycin, nalidixic acid, rifampicin, gentamicin, chloramphenicol, tetracycline, and ampicillin were tested using a continuous flow chemostat system mimicking the colonic environment. A mix of three strains of *E. coli* (with and without

R-plasmid and F'lac plasmid) with similar MICs for the antibiotics in study was added to the system. Tetracycline at 0.25 μg/mL (1/10 of the MIC of the susceptible strain) favored the growth of the resistant strain; however, no R-plasmid transfer was observed. Similar results were seen with chloramphenicol and gentamicin (24).

One of the FDA research contracts used a continuous flow one chambered-chemostat system inoculated with human intestinal microflora to study the effect of low doses of tetracycline, neomycin, erythromycin, and ciprofloxacin on bacterial populations, disruption of the barrier effect, metabolic activity, and development of resistant strains. Under the conditions tested, ciprofloxacin at dose levels of 0.43, 4.3, and 43 μg/mL produced a dose-dependent decrease in *E. coli* population. A decrease in the population of *Bacteroides* was seen with 43 and 4.3 μg of ciprofloxacin per mL of chemostat medium, and a decrease in susceptibility of *Bacteroides* to 4 μg/mL of ciprofloxacin was found in the 0.43 μg/mL chemostat. Disruption of the barrier effect was evidenced by colonization of the 4.3 and 5 μg of ciprofloxacin per mL chemostats challenged with a strain of *Salmonella kedogou* (25, 26). Tetracycline produced a transitory dose-dependent increase in resistant *E. coli* strains at dose levels of 0.15, 1.5 and 15 μg/mL. Neomycin changed the proportion of short chain fatty acids at 1.78, 17.8, and 178 μg/mL, produced a dose-dependent decrease in the metabolism of bile acids, a dose-dependent decrease in azoreductase activity, and a significant increase in the percentage of resistant enterococci at 17.8 and 178 μg/mL. Erythromycin showed a dose-dependent transitory decrease in bile acid metabolism similar to neomycin, at 1.5, 15, and 150 μg/mL (27).

Sarafloxacin tested in an *in vitro* model simulating the colonic conditions and in broth culture inhibited the growth of *E. coli* in a dose-dependent manner at 0.24 and 3.7 μg/mL. The drug was less inhibitory in the model than in broth culture. The authors concluded that *Bacteroides* and *Bifidobacterium* were rather insensitive to the drug in this model (28).

The effect of low levels of antibiotics has also been studied in human flora-associated rodents. Tilmicosin at 400 μg/kg/day produced a transient increase in the number and proportion of enterobacteria and spiramycin at 500 μg/kg/day showed a significant increase in the number of spiramycin-resistant enterobacteria when given to human flora-associated rats for 5 days (29). Low doses of ampicillin, colistin, flumequin, gentamicin, tetracycline, or streptomycin given orally for 2 weeks to germ-free mice colonized by two isogenic strains of *E. coli*, (one carrying an R-plasmid) showed a strong correlation between antimicrobial dose and selection of resistant *E. coli* strains (30). Human flora-associated mice continuously administered low doses of ampicillin (0.5 μg/mL), chlortetracycline (0.5 μg/mL), or streptomycin in the drinking water showed an increase in the number of resistant *E. coli* (22).

Under the FDA research contracts, the effects of residue levels of tetracycline, neomycin and ciprofloxacin were also studied in human flora-associated mice. Tetracycline at 1, 10 and 100 ppm in the drinking water for 8 weeks produced a significant increase in resistant enterococci and *Bacteroides fragilis* at all dose levels and *Enterobacteriaceae* at 10 and 100 ppm. The effect disappeared after cessation of treatment. The barrier effect

to a challenge strain of *Salmonella scharzendrung* was also impaired at 100 ppm. Neomycin at 0.2, 2, and 20 ppm in the water did not produce any significant effect on the endpoints evaluated (counts of susceptible and resistant target bacteria, metabolic activity parameters and colonization resistance properties). Ciprofloxacin at 1, 10, and 100 ppm produced a significant decrease in total aerobes and enterococci populations. Enterobacteria decreased in a dose-dependent manner with total elimination at 10 and 100 ppm of the drug. The percentage of resistant enterococci and resistant clostridia increased during treatment with 100 ppm of ciprofloxacin. Resistant *Bacteroides fragilis* increased at 10 and 100 ppm. The barrier effect against a strain of *Salmonella typhimurium* was disrupted with 100 ppm of the drug, but the effects were not clear with 10 and 1ppm (31, 32, 33, 34, 35, 36).

C. Endpoints of Public Health Concern

The main adverse effects of antimicrobial drugs on the human intestinal microflora are 1) alterations of the metabolic activity of the intestinal microflora, 2) development of resistant strains, 3) disruption of the barrier effect with overgrowth of potentially pathogenic microorganisms, and 4) changes in bacterial populations.

Colonization resistance or barrier effect

The barrier effect (or colonization resistance) is the property of the flora that prevents overgrowth of transient potentially pathogenic microorganisms, the outgrowth of indigenous potentially pathogenic microorganisms, and/or proliferation of antibiotic-resistant strains. The barrier effect may be disrupted by the action of any antimicrobial drug on the intestinal microflora. This property is associated mainly with the indigenous anaerobic bacteria (6). A classic example of disturbance of the intestinal microflora is that caused by clindamycin. Clindamycin is an antibiotic with activity against Gram-positive cocci and many anaerobic bacteria such as *Bacteroides fragilis, Fusobacterium, Peptostreptococcus, Peptopcoccus*, and *Clostridium perfringens*. Diarrhea (at different degrees) associated with clindamycin treatment has a frequency of 2-20% (37). A severe syndrome, pseudomembranous colitis, caused by a *Clostridium difficile* toxin has been described in up to 10% of the patients treated with this antibiotic. The syndrome may be fatal if not treated (37). Although *Clostridium difficile* colitis was initially associated with clindamycin, ampicillin and cephalosporins have also been cited as common causes of this syndrome (38).

Overgrowth of transient pathogenic microorganisms or commensal organisms of the intestinal microflora, due to disruption of the barrier effect, is an effect that was linked to antibacterial drugs many years ago. This overgrowth may result in enteric infections such as staphylococcal enterocolitis, infections due to *Salmonella, Klebsiella, E.coli, Pseudomonas, Proteus, Yersinia enterocolitica*, and others. Studies performed in volunteers have shown that amoxicillin, cefotaxime, clindamycin, or co-trimoxazole disrupt the barrier effect and facilitate colonization of challenge strains of *K. pneumonia* and *E. cloacae*. An increase in the number of resistant Gram-negative bacteria, enterococci, and yeasts was also seen in the feces of the volunteers (39, 40, 41, 42).

Disruption of the barrier effect resulting in increased populations of normal flora components such as enterococci and *Clostridium perfringens* may also produce antibiotic-induced diarrhea (43).

Selection of resistant strains

Low or subtherapeutic doses of antimicrobials can increase the percentage of resistant microorganisms in the normal intestinal microflora. Studies have shown that healthy people can harbor a large number of antibiotic resistant bacteria in the intestinal flora, mainly of the *Enterobacteriaceae* family (almost 40% of people continuously carry resistant bacteria, although the numbers and types vary daily) (4). Drug-resistant commensals bearing R-plasmids could also increase in number due to low doses of antimicrobial drugs favoring plasmid transfer to pathogenic bacteria and the addition of new genes on a pre-existing R-plasmid. *Escherichia coli*, a normal resident of the intestinal microflora, may be resistant to multiple antimicrobial drugs and may be important reservoirs of resistant plasmids from which genes coding for resistant determinants may be transferred to human pathogens (44, 45).

There are different forms of resistance selection in the intestinal microflora. One is the selection of species that are intrinsically less susceptible than others, such as *Clostridium difficile*, yeasts, various Gram-negative species, and Enterococcus. Another form is the selection of mutants from normally susceptible strains that exhibit decreased susceptibility to antimicrobials. These mutations may occur in genes that regulate different functions of the bacterial cell such as outer membrane proteins involved in the diffusion of antibiotics across the bacterial cell wall or the regulation and expression of beta-lactamases. Multiple antibiotic resistant phenotypes may arise by movement of mobile genetic elements such as plasmids or transposons that can carry resistance genes. This transposition may occur in the absence of antibiotic selective pressure, but antibiotics have been shown to increase transposition. For example, the transmission of tetracycline resistance in Gram-positive bacteria by a conjugated transposon is stimulated by sub-inhibitory concentrations of tetracycline (46).

The cost of resistance can be measured in terms of increased morbidity (extended length of therapy or hospitalization), mortality (death due to infections with antibiotic resistant strains), and cost of therapy. Unrecognized costs associated with antibiotic resistance include persistence of resistance in a population or in a patient. Resistance may or may not decrease when the use of an antibiotic is suspended (46).

Alteration of the metabolic activity of the intestinal microflora

The intestinal microflora catalyzes a number of reactions including hydrolysis, reductions, degradations, and synthesis. The biotransformation of compounds may be beneficial or have adverse toxicological consequences for the host.

Antimicrobial drugs may alter the ecological balance of the flora resulting in alteration of its biotransformation capacity to change the original activity or toxicity of compounds (1,

4). Indicators of the metabolic activity of the intestinal microflora include measurement of hydrolytic enzymes (beta-glucosidades, beta-glucuronidase), reductases (nitroreductase, azoreductase, nitrate reductase), metabolism of bile acids and cholesterol, production of short chain fatty acids, determination of cellular fatty acids, sulfate reduction, and others. The assessment of these activities is indirectly related to the barrier effect, although the observed changes cannot always be related to public health concerns (1, 44, 47).

The effect of changes in the metabolic activity of the flora will depend on the specific activity and the physiological or pathological conditions with which it has been associated. For example, evaluation of bile acids metabolism by the intestinal microflora may indicate drastic changes in flora composition because primary bile acids are metabolized by the flora to secondary bile acids and excreted or passively absorbed and re-circulated. The ratio of primary to secondary bile acids may indicate profound changes in bacterial composition. Secondary bile acids metabolites have been shown to be cancer promoters, therefore, an increase in the ratio of secondary bile acid metabolites may increase the risk of colon cancer for the susceptible individuals. In addition, bile acids have been associated with an increase in serum cholesterol (48).

Cellular fatty acids also indirectly relate to changes in the proportion of colonic species. Short chain fatty acids, present in high concentrations in the colon and in feces, are end-products of microflora metabolism. Changes in the molar ratios of short chain fatty acids due to the effect of antimicrobial drugs may also indicate changes in bacterial populations (4, 49). Drugs such as ampicillin, clindamycin, vancomycin and bacitracin reduce the levels of fecal short chain fatty acids (48). Therapeutic doses of bacitracin and vancomycin have been shown to drastically reduce fecal short chain fatty acid excretion while doxycycline, nalidixic acid, ofloxacin, and co-trimoxazol have little or no effect. Erythromycin has a moderate effect on fecal short chain fatty acids excretion. Other antimicrobial drugs can elevate the levels of a specific fatty acid (49). The reduction of cholesterol to coprostanol by intestinal bacteria is another way to monitor the stability of the intestinal microflora. However, human populations vary considerably in their degree of cholesterol reduction (48).

The level of enzymes in feces represents potential bacterial activity of the microflora. Conjugated compounds and complex polysaccharides are metabolized in the colon by bacterial glycosidases (beta-glucuronidase being the most important one). The beta-glucuronidase activity in the colon is mainly the responsibility of the *Bacteroides* species. Changes in its activity may result in changes in the capacity of the microflora to influence the pharmacokinetics of drugs, genotoxins, tumor promoters, and other bioactive compounds. Another hydrolytic enzyme, beta-glucosidase, hydrolizes a broad range of sugar conjugates. Several bacteria produce this enzyme, such as the streptococci and lactobacilli. However, since these species are not present in high numbers in the colon, the majority of the beta-glucosidase activity is again the responsibility of the *Bacteroides* group. Of the reductase enzymes, azoreductase activity is also responsible for reduction of azo dyes (48).

4). Indicators of the metabolic activity of the intestinal microflora include measurement of hydrolytic enzymes (beta-glucosidades, beta-glucuronidase), reductases (nitroreductase, azoreductase, nitrate reductase), metabolism of bile acids and cholesterol, production of short chain fatty acids, determination of cellular fatty acids, sulfate reduction, and others. The assessment of these activities is indirectly related to the barrier effect, although the observed changes cannot always be related to public health concerns (1, 44, 47).

The effect of changes in the metabolic activity of the flora will depend on the specific activity and the physiological or pathological conditions with which it has been associated. For example, evaluation of bile acids metabolism by the intestinal microflora may indicate drastic changes in flora composition because primary bile acids are metabolized by the flora to secondary bile acids and excreted or passively absorbed and re-circulated. The ratio of primary to secondary bile acids may indicate profound changes in bacterial composition. Secondary bile acids metabolites have been shown to be cancer promoters, therefore, an increase in the ratio of secondary bile acid metabolites may increase the risk of colon cancer for the susceptible individuals. In addition, bile acids have been associated with an increase in serum cholesterol (48).

Cellular fatty acids also indirectly relate to changes in the proportion of colonic species. Short chain fatty acids, present in high concentrations in the colon and in feces, are end-products of microflora metabolism. Changes in the molar ratios of short chain fatty acids due to the effect of antimicrobial drugs may also indicate changes in bacterial populations (4, 49). Drugs such as ampicillin, clindamycin, vancomycin and bacitracin reduce the levels of fecal short chain fatty acids (48). Therapeutic doses of bacitracin and vancomycin have been shown to drastically reduce fecal short chain fatty acid excretion while doxycycline, nalidixic acid, ofloxacin, and co-trimoxazol have little or no effect. Erythromycin has a moderate effect on fecal short chain fatty acids excretion. Other antimicrobial drugs can elevate the levels of a specific fatty acid (49). The reduction of cholesterol to coprostanol by intestinal bacteria is another way to monitor the stability of the intestinal microflora. However, human populations vary considerably in their degree of cholesterol reduction (48).

The level of enzymes in feces represents potential bacterial activity of the microflora. Conjugated compounds and complex polysaccharides are metabolized in the colon by bacterial glycosidases (beta-glucuronidase being the most important one). The beta-glucuronidase activity in the colon is mainly the responsibility of the *Bacteroides* species. Changes in its activity may result in changes in the capacity of the microflora to influence the pharmacokinetics of drugs, genotoxins, tumor promoters, and other bioactive compounds. Another hydrolytic enzyme, beta-glucosidase, hydrolizes a broad range of sugar conjugates. Several bacteria produce this enzyme, such as the streptococci and lactobacilli. However, since these species are not present in high numbers in the colon, the majority of the beta-glucosidase activity is again the responsibility of the *Bacteroides* group. Of the reductase enzymes, azoreductase activity is also responsible for reduction of azo dyes (48).

Changes in bacterial populations

Drastic changes in bacterial populations by antimicrobial drugs may disrupt the colonization resistance properties of the intestinal microflora, the metabolism of compounds that undergo enterohepatic circulation (estrogens, vitamins, cholesterol, protoporphyrin, and bile acids), or the metabolism of drugs undergoing enterohepatic circulation resulting in increasing blood levels of the drugs (4, 44). For example, therapeutic doses of tetracycline and erythromycin reduce the population of *Eubacterium* in the colon, which is responsible for the reduction of digoxin, a cardioglycoside drug. Dangerous blood levels of digoxin may be reached in patients treated with these antibiotics (4). Antimicrobial drugs can also influence estrogen metabolism by eliminating intestinal bacteria responsible for their deconjugation and reabsorption of the free hormone. The result is an increase in the fecal excretion of conjugated estrogens. In addition, the contraceptive effect of synthetic steroids may be diminished by the effect of antibiotics due to changes in the intestinal microflora that result in alteration of the metabolism of the chemicals and a decrease in the circulating half-life of the estrogen dose (4).

D. Model Systems for Evaluating Endpoints of Concern

In vitro and *in vivo* tests and model systems have been used to study the effects of antimicrobial drugs on the human intestinal microflora. These models attempt to simulate the human colon and its microbial population.

MIC data

Quantitative *in vitro* antimicrobial susceptibility testing on bacteria from the colonic flora has been used by pharmaceutical sponsors and presented to international organizations such as JECFA and the CVMP for the assessment of the human food safety of veterinary antimicrobial drug residues. These data, presented as MICs, have been incorporated into formulas for establishing the ADI for antimicrobial veterinary drug residues in food. The advantages of determining MICs are simplicity, rapidity, and low cost. However, use of this testing for assessing the human food safety of veterinary antimicrobial drug residues in the food from treated animals has disadvantages. Some of the disadvantages of MIC determinations for this purpose are the following: (1) because they are done on pure bacterial cultures, they are not representative of the ecological system in the human intestinal tract; (2) they do not take into account the representativeness of the bacteria studied, the pH, anaerobic conditions of the colon, or *in vivo* conditions such as absorption, metabolism, enterohepatic circulation, and fecal concentration of the drug; (3) they do not assess long-term effects of antimicrobials on the intestinal microflora; (4) they do not allow quantitation of minor populations of resistant bacteria (not enough selected clones); (5) they do not assess perturbations of the intestinal microflora such as disruption of the barrier effect and changes in enzyme function (50, 51, 52, 53).

In vitro model systems

Static batch, semi-continuous, and continuous flow culture systems mimicking the colonic environment have been developed for studying the effects of diets, food additives, and drugs on the intestinal microflora. Static (batch) cultures are useful for performing short-term metabolism studies and for determining the potential of a drug of being inactivated due to binding or chemical transformation. However, the bacterial composition changes with time. Semi-continuous and continuous culture systems include chemostat culture systems inoculated with one or more types of bacteria or with feces to which fresh medium is added and used; culture media is removed periodically or continuously, depending on the model. Different models have been developed, ranging from a single vessel to a two or three-stage model. The advantage of these models is that they model the intestinal microflora and allow the study of long-term exposure to different drug concentrations. These systems can be used for determining NOELs for microbiological endpoints (functional endpoints, resistance emergence, and barrier effect). However these models do not take into account host metabolism, the bacterial populations are still lower than those in the colon, and expertise is required to set up and maintain the systems. Studies using a semi-continuous culture system have also shown high variability for determining the NOELs in colonization resistance studies using human intestinal microflora. These variations could be due to fecal inoculum differences or other factors (47, 53, 54).

Simulated gut models mimicking the passage of food through the human gastrointestinal tract have been developed. In such models, the test substance is incubated sequentially under conditions similar to the stomach and the intestine, bacteria are added to the medium, and survival is determined by microbiological plate counts. This model is relatively inexpensive and simple to perform; however, it does not resemble the complexity of the intestinal microflora and does not account for host metabolism. The endpoint to be studied is survival of indicator bacteria from the gut (53).

A gastrointestinal simulation model was also developed for determining NOELs and establishing ADIs for antimicrobial drug residues based on MIC values for indicator bacteria. The indicator bacteria are checked for changes in MIC due to exposure to low concentrations of antimicrobial drugs (55). A similar model was used to study the effect of sarafloxacin on *E.coli*, *Bacteroides fragilis*, and *Bidifobacteria* strains (56). Another similar model was developed for studying the effect of antibiotic drug residues on intestinal microflora under anaerobic conditions. The model is inexpensive, easy to set up and studies drug exposure in an intermittent manner providing some insight on the interaction between the colonic conditions, residue levels of drugs and the resultant antimicrobial activity (57). Although this model takes into account some aspects of host metabolism in the colon that are not considered in the standard MIC test, the model has all the disadvantages of pure culture testing described above under "MIC data" (53).

A semi-continuous culture system was developed for maintaining the human colonic microflora and studying their interaction and fermentation processes for a long period of time (81 days). The authors concluded that the model could be suitable for studying microbial activities and bacterial populations of the colon (58). A semi-continuous flow chemostat system inoculated with human intestinal microflora was used to study the

activity of the microflora on the metabolism of three different chemical classes of xenobiotics (59). The same model was also used to determine if the metabolic activity of the flora could be maintained *in vitro*. The model proved to be useful for maintaining the diverse population of the colon and the metabolic activity of the flora for prolonged periods of time (60). The semi-continuous model has also been studied as a possible model for determining NOELs for antimicrobial drug residues based on the disruption of the barrier effect of the intestinal microflora. The model was capable of detecting a dose-response effect to clindamycin. However, the responses varied among experimental runs and it was concluded that further studies were needed to investigate the causes of variability before determining the usefulness of this model for assessing barrier effect for regulatory purposes (54).

A continuous flow chemostat model has been used to study interactions between representative strains of the human colon and strains of seven enteropathogenic bacteria (61). The same model, inoculated with feces from human volunteers, was used for studying the function of the colonic bacteria through time and proved to be able to maintain actively fermenting viable cultures for at least 21 days (62). A three-stage continuous culture system inoculated with mixed populations of human intestinal bacteria was developed to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis. Each stage represented a section of the large intestine with specific nutritional and pH characteristics. The model was able to sustain bacterial growth for 120 days and it was appropriate for these types of studies (63).

The effect of subinhibitory concentrations of streptomycin sulfate, nalidixic acid, rifampicin, gentamicin, chloramphenicol, tetracycline, and ampicillin on the development of resistant *E. coli* strains was studied utilizing a continuous flow chemostat system (64). The FDA funded research also studied the applicability of a continuous flow one-chambered chemostat inoculated with feces from healthy volunteers for determining the effects of low doses of tetracycline, neomycin, erythromycin, and ciprofloxacin on various mirobiological endpoints of the human intestinal microflora (see section B above). Data from preliminary studies performed to establish the chemostats and develop the assays for the different endpoints are described in TechLab reports (65, 66, 67, 68, 69, 70). The detailed results of this research can be found in the reports of the FDA contract for each drug (25, 26, 27, 71, 72, 73, 74,75).

In vivo model systems

Human volunteers, beagle dogs, pigs, conventional rodents, gnotobiotic rodents, and human flora-associated rodents have been used to study the effect of substances on the intestinal microflora. The human model is the most appropriate model. However, while the human model is very useful for clinical studies, toxicological studies are not possible because there are ethical considerations and the number of volunteers is a limiting factor for the power of the studies. The advantage of using conventional animals is that the flora can be monitored at different sections of the intestinal tract, dietary environmental factors can be controlled, and many microbiological endpoints can be evaluated. However, extrapolating the findings to the human is problematic because of differences

in bacteria composition and metabolism. Gnotobiotic animals are useful for studying host-bacteria or bacteria-bacteria interactions. However, their relevance to the normal human ecosystem is questionable. Rodents (rats or mice) inoculated with diluted human feces are suitable for performing studies on human microflora interactions and metabolism because the implanted flora retains many of the characteristics of the human flora. These models are easy to control, a large number of subjects can be used to obtain statistical power for the studies, and metabolic and ecological interactions of the flora, as well as colonization resistance to challenge bacteria, can be studied. However, they are expensive, difficult to set up and maintain and the gut physiology of the animal may not be similar to that of humans (47, 52, 53).

Studies on the effect of low doses of antimicrobials on human volunteers have shown that oxytetracycline produced a transient effect on the emergence of resistant coliform bacteria (20) and also increased the number of resistant *Enterobacteriaceae* in the fecal microflora of adult volunteers (21). Ampicillin and streptomycin given orally to human volunteers at a dose of 15 mg/day of each drug for 21 days significantly raised the concentration in the feces of *E. coli* resistant to ampicillin, chlortetracycline and streptomycin. The dose of 1.5 mg ampicillin gave less significant results; the increase in the population of resistant *E. coli* occurred in only two volunteers (22).

Beagle dogs have been used to study the effect of low levels of oxytetracycline, dihydrostreptomycin, virginiamycin, and penicillin on the emergence of resistant strains in the intestinal microflora. Oxytetracycline at 10 μg/g diet for 44 days produced a shift from a predominantly drug-susceptible population of enteric lactose-fermenting bacteria to a multiple antibiotic resistant population. No shift was observed with 2 μg/g (76). Dihydrostreptomycin at 2 and 10 μg/g diet produced shift from a streptomycin-susceptible to a streptomycin resistant coliform fecal population (77). Virginiamycin at 55 μg/g diet resulted in an increase in the proportion of lactose-fermenting bacteria in the feces that were resistant to ampicillin, dihydrostreptomycin, tetracycline, or chloramphenicol. Penicillin at 110 μg/g diet had a similar effect (78). The power of the tests was limited because of the inter-animal and day-to-day variability of the flora.

Germ-free rodents inoculated with specific bacterial strains of the human intestinal microflora (*E. coli* strains with and without a tetracycline-resistant plasmid) have been used to study bacteria-bacteria interactions due to minimal doses of antimicrobial drugs. The results showed that *in vivo* interactions between the three strains were very different from those obtained *in vitro*, with the tetracycline-resistant strain becoming dominant within one day and replacing the sensitive *E. coli*. *In vitro* studies had shown that the *E. coli* resistant strains were repressed by the sensitive strains. It is concluded that *in vivo* studies should be performed for the determination of no-effect levels of antibiotic residues in the gut (79). This model has also been used to study the effect of minimum doses of antibiotics ampicillin, colistin, flumequin, gentamicin, tetracycline, and streptomycin) on the selection of a resistant plasmid in germ-free mice colonized with two isogenic strains of *E. coli* (one carrying a resistant plasmid). The antibiotics were given to mice in the drinking water at dose levels ranging from 0.9 to 12.8 μg/mL. Ampicillin, gentamicin, tetracycline, and streptomycin increased the number of resistant

bacteria. The minimum selecting level for resistance was defined as the drug concentration producing a 10-fold increase in the resistant population compared with that of the inoculum and to a 100-fold increase compared with the control group (30). This model does not represent the intestinal ecosystem in which many types of microorganisms coexist and interact with each other.

Pig flora-associated mice (mice implanted with fecal flora from pigs) have been used to study the effect of low doses of bambermycin (5 μg/mL of drinking water), carbadox (50 μg/mL), chlortetracycline (20 μg/mL, and olaquindox (50 μg/mL) on the emergence of resistant coliforms. The occurrence of drug-resistant coliforms was higher in mice given chlortetracycline and lower in mice dosed with bambermycins; olaquindox and carbadox did not change the proportion of resistant coliforms in mice feces (80). The model was recommended for studying the development of resistant strains due to low doses of antimicrobials using other animals' flora or human intestinal flora.

Germ-free rodents colonized with human intestinal microflora is the *in vivo* model most studied to date because it incorporates the gross bacterial composition of the flora, the barrier effect, and other functions of the microflora in the human. The human flora-associated mice (HFA mice) model has been used extensively to study the effects of antimicrobials on microflora composition and on resistance to pathogen challenge (44). Germ-free mice inoculated with human flora and with fecal anaerobes were used to study the gross composition of the flora before and after implantation in the mice and the barrier effect of the anaerobes against *Pseudomonas aeruginosa* in immunocompromised mice. The gross composition of the flora before and after implantation was similar and remained stable after five weeks. In addition, the implanted flora and the anaerobes induced an antagonistic effect against *E. coli*. In contrast to the complete flora, the anaerobes were not invasive in immunosuppressed mice and induced colonization resistance and antagonism against *Pseudomonas aeruginosa* (81). The same model has been used to study the effect of erythromycin (dose levels of >1,000 μg/g in the human donor and in mice) on the barrier effect of the human flora. The drug did not reduce colonization resistance to *Candida albicans, Clostridium perfringens*, and erythromycin-sensitive *E. coli*; however, it reduced at some degree colonization resistance against *Pseudomonas aeruginosa, Clostridium difficile* and erythromycin-resistant *E. coli* (82). The effect of high doses of nifurzide and nifuroxazide on bacterial populations and on the colonization resistance to enterotoxigenic *E. coli* and *Shigella flexeri* was also studied in human flora-associated mice. Nifurzide significantly reduced colonization resistance to *E. coli* and Shigella flexeri (83). The effect of norfloxacin on the colonization resistance properties of the human intestinal microflora was studied in in HFA-mice. Resistance to colonization by exogenous bacteria was reduced for 2/14 of the strains tested (*Pseudomonas aeruginosa* and *Candida albicans*) (84). More recently, the HFA-mice model was evaluated as a model for studying persistence of the human flora in the gnotobiotic mice, the metabolic activity of the flora, and the colonization resistance to a *Salmonella typhimurium* strain. The model seemed appropriate for studying colonization resistance properties of the flora, since a *Salmonella typhimurium* strain could not be established in the HFA-mice but did invade the intestine of a germ-free mice (85). HFA-rats have also been studied to evaluate the potential effect of low doses of tilmicosin and

spiramycin on bacterial composition and on the development of resistant strains. Low levels of tilmicosin and spiramycin given orally to HFA-rats for 5 days showed no major changes in the anaerobe population but the number of spiramycin resistant enterobacteria increased significantly from day 2 (29). This model had been used earlier to study the formation of apparent total N-nitroso compounds in the human intestinal microflora of implanted rats (86).

The FDA funded research studied the effect of low doses of tetracycline, neomycin and ciprofloxacin on the human intestinal microflora using the HFA-mice model. The microbiological endpoints evaluated in the studies were similar to those evaluated in the *in vitro* chemostat studies discussed in B above. Detailed results of these studies are found in the quarterly reports to the FDA (31, 32, 33, 34, 35, 36, 87, 88).

E. International Approaches for the Regulation of Antimicrobial Drug Residues in Food

The safety of antimicrobial residues in food have been assessed internationally by three organizations: 1) the Codex Alimentarius Commission (CAC); 2) the European Medicines Evaluation Agency (EMEA); and 3) International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH). Each organization has scientific groups that provide advise on the safety of veterinary drug residues and appropriate studies to determine their safety. The scientific advisory groups make recommendations that will later become standards when approved by the organizations. The CAC sets standards for veterinary drug residues based on recommendations made by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) through the Codex Committee on Residues of Veterinary Drugs in Food. The EMEA sets standards based on recommendations from the Committee for Veterinary Medicinal Products (CVMP). The VICH recommends requirements and protocols for determining human food safety of veterinary drugs based on recommendations from the Safety Working Group (SWG).

Joint Expert Committee on Food Additives (JECFA)

JECFA initially addressed the microbiological safety of veterinary drug residues in foods in June of 1987. The Committee concluded that the antimicrobial properties of veterinary drug residues would become the determining factor in safety evaluation when the toxicity of the substance is so low that their residues could be tolerated without any withdrawal period. In such a case, the safety of the residues would be based on their danger to human health due to their selective pressure on the intestinal microflora favoring growth of microorganisms with natural or acquired resistance (89).

In 1990 the Committee concluded that the most important characteristics of the intestinal microflora to be considered when assessing the microbiological risk of antibiotic residues in food are the proportion of anaerobic bacteria, the stability of the flora, and the barrier effect. Thus, the Committee determined that the safety evaluation of antimicrobial residues should be based on data related to bacteria that constitutes the flora, taking into account the barrier effect. If human data are not available, animal studies might be considered. The Committee encouraged the validation of animal models such as

haloxenic rodents implanted with human intestinal microflora and also concluded that, in the absence of *in vivo* data, *in vitro* data such as MIC's could be used on a temporary basis for safety evaluations (90).

In 1991, JECFA for the first time calculated the ADI for an antimicrobial drug (spiramycin) using MIC data from four species of the dominant anaerobic flora. A formula was developed using the modal MIC of the bacteria tested, safety factors to cover to different variables, the daily fecal bolus, the fraction of oral dose available, and the weight of humans (91).

In 1994, JECFA concluded that the evidence of risk due to low levels of antimicrobial residues is minimal and other methods for studying the microbiological endpoints may be useful for assessing this risk. MIC data would continue to be accepted for determining ADIs until other methods could be developed and accepted for this purpose (92).

In 1995, JECFA discussed a new 'decision tree' approach to the safety evaluation of antimicrobial residues (93), and in 1996 the Committee concluded that more research was needed concerning the public health risk of antimicrobial residues and their effects on the human intestinal microflora. They recommended that MIC data should not be the only method used to calculate an ADI and that data from *in vitro* or *in vivo* model systems or any other relevant data should be used for setting ADIs. In absence of human data, data from *in vivo* model systems (e.g., human flora-associated rodents) or *in vitro* models (e.g., continuous flow cultures) could be used for determining ADIs for antimicrobial drugs. They recognized the limitations of the formula method, and the formula using MIC data was again modified. The Committee recommended the development and validation of *in vitro* and *in vivo* model systems that would be more appropriate for determining NOELs and setting ADIs for antimicrobial residues. The Committee also concluded that, when sufficient data are available, no additional microbiological information on drug effects on the human intestinal microflora would be required if the residues in food do not exceed 1.5 mg/person/day (94).

In 1998, the Committee determined the ADI of several antimicrobial agents (gentamicin, sarafloxacin, tetracyclines) based on effects on *in vitro* studies and using the formula approved in their 47th meeting. The tetracyclines' ADI was determined based on the development of resistant *E. coli* seen in a human study and also confirmed in a continuous flow chemostat study (95). In February 2000, the Committee determined the ADI for lincomycin using the 'decision tree' approach discussed in 1995.

Committee for Veterinary Medicinal Products (CVMP)

The CVMP began evaluating the human food safety of veterinary drug residues in 1995. Their primary focus on microbiological risk is the assessment of effects and safety of antimicrobial drug residues on the human intestinal microflora (96).

Initially, the CVMP applied the approach followed by JECFA in 1992 for the evaluation of antimicrobial drug residues in food. However, some difficulties were encountered and

915 in March 1994, the CVMP adopted a guideline that would be used for the next five years
916 until further review of the approach. Three evaluation approaches are accepted by the
917 CVMP: human data with an appropriate safety factor; data to demonstrate the no-
918 observed effect level (NOEL) obtained in (HFA) rodents when the induction of resistance
919 and reduction of the barrier effect are studied; or the calculation of a microbiological ADI
920 from *in vitro* MIC data obtained under conditions similar to those in the colon. The
921 CVMP developed a formula slightly different than the JECFA formula.
922
923 In April of 2001, the CVMP published for consultation a revised guideline entitled
924 "Revised Guideline on Safety Evaluation of Antimicrobial Substances Regarding the
925 Effects on Human Gut Flora". The revised guideline states that the current CVMP
926 microbiological ADI formula will continue to be used as an interim measure until the
927 adoption of a harmonized VICH guideline. The approaches to calculate the ADI remain
928 the same; however, the formula including MIC data was slightly modified. The revised
929 guideline states that the two endpoints of concern that should be addressed in the
930 determination of a microbiological ADI are reduction or elimination of the barrier effect
931 of the normal flora and development of an/or increase in the pool of antibiotic-resistant
932 strains of potentially pathogenic microorganisms.
933
934 The CVMP calculates both a toxicological and a microbiological ADI for antimicrobial
935 drugs. The most relevant ADI (usually the lowest) is used to determine the maximum
936 residue limit (MRL) (97).
937
938 International Cooperation on Harmonisation of Technical Requirements for Registration
939 of Veterinary Medicinal Products
940 The VICH, a trilateral program with representatives from the European Union, the United
941 States, Japan, and attendance from Australia/New Zealand, initially addressed the safety
942 of antimicrobial drug residues in April of 1999. At that time, the VICH SWG agreed to
943 charge a sub-group of experts with attending the 1999 FDA workshop "Microbiological
944 Safety of Drug Residues in Food" and writing recommendations to the SWG on the
945 regulation of antimicrobial residues based on effects on the human intestinal microflora.
946 The expert group recommended that a Task Force be formed with microbiology experts
947 in human intestinal microflora ecology. The Task Force would review all information
948 available and make recommendations to the SWG on testing methods and protocols for
949 determining NOELs for antimicrobial drug residues based on effects on human intestinal
950 microflora. The Microbial Safety Task Force has met twice (in July of 2000 and in May
951 of 2001) and is working to complete a mandate of the SWG concerning recommendation
952 on testing methods and protocols for the safety evaluation of antimicrobial drug residues
953 in food.

REFERENCES

1. Carman, R.J., Van Tassell, M.S., and Wilkins, T.D. The Normal Intestinal Microflora: Ecology, Variability and Stability. Veterinary and Human Toxicology 35 (Supplement 1): 11-14, 1993.
2. Edlund, C. and Nord, C.E. Effect of Quinolones on Intestinal Ecology. Drugs 58 (Supplement 2): 65-70, 1999.
3. Finegold, S.M., Mathisen, G.E., and George, W.L. Changes in Human Intestinal Flora Related to the Administration of Antimicrobial Agents. In: *Human Intestinal Microflora in Health and Disease*. Academic Press, Inc. ISBN 0-12-341280-3. pp 355-429, 1983.
4. Gorbach, S.L. Perturbation of Intestinal Microflora. Veterinary and Human Toxicology 35(Supplement 1): 15-23, 1993.
5. Heimdahl, A., Kager, L., and Nord, C.E. Changes in the Oropharyngeal and Colon Microflora in Relation to Antimicrobial Concentrations in Saliva and Feces. Scand. J. Infect. Dis. Supplement 44: 52-58, 1985.
6. Vollaard, E.J. and Clasener, H.A.L. Minireview. Colonization Resistance. Antimicrob. Agents Chemother. 38 (3): 409-414, 1994.
7. FDA/CVM. Guidance. Microbiological Testing of Antimicrobial Drug Residues in Food. January, 1996.
8. Fernández, A.H. Personal communication. FDA Proposal for Establishing Microbiological ADIs for Antimicrobial Residues. Workshop "Microbiological Safety of Drug Residues in Food". Rockville, MD, September 20-21, 1999.
9. Center for Veterinary Medicine, CVM Update, CVM Plans to Modify Guidance on Microbiological Safety of Drug Residues in Food, March 3, 2000, http://www.fda.gov/cvm/fda/infores/updates/52up.html, 28 September 2000.
10. Wilkins, T.D. Personal Communication. Advantages and Disadvantages of *In Vitro* Model Systems to Set ADIs. Workshop "Microbiological Safety of Drug Residues in Food". Rockville, MD, September 20-21, 1999.
11. Saxerholt, H., Carlstedt-Duke, B., Hoverstad, T., Lingaas, E., Norin, K.E., Steinback, M., and Midtvedt. T. Influence of Antibiotics on the Faecal Excretion of Bile Pigments in Healthy Subjects. Scand. J. Gastroenterol. 21: 991-996, 1986.
12. Brumfitt, W., Franklin, I., Grady, D. et al. Changes in the Pharmacokinetics of Ciprofloxacin and Fecal Flora During Administration of a 7-day Course to Human Volunteers. Antimicrob. Agents Chemother., 26: 757-761, 1984.
13. Rozenberg-Arska, M., Dekker, A.W., Verhoef, J. Ciprofloxacin for Selective Decontamination of the Alimentary Tract in Patients with Acute Leukemia During Remission Induction Treatment: The Effect on Fecal Flora. J. Infect. Dis. 152: 104-107, 1985.
14. Terg,, R., Llano, K., Cobas, S.M., Brotto, C., Barrios, A., Levi, D., Wasen, W., and Bartellini, M.A. Effects of Oral Ciprofloxacin on Aerobic Gram-negative Fecal Flora in Patients with Cirrhosis: Results of Short- and Long-term Administration, with Daily and Weekly Dosages. Journal of Hepathology 29: 437-442, 1998.
15. Inagaki, Y. and Yamamoto, N. The Effect of DU-6859a, a New potent Fluoroquinolone, on Fecal Microflora in Human Volunteers. Jpn. J. Antibiot. 3: 368-379, 1995.

16. Nord, C.E. and Edlund, C. Ecological Effects of Antimicrobial Agents on the Human Intestinal Microflora. Microbial Ecol. Health Dis. 4: 193-207, 1991.
17. Nord, C.E., Heimdahl, A ., and Kager, L. Antimicrobial Induced Alterations on the Human Oropharyngeal and Intestinal Microflora. Scand. J. Infec. Dis., Supplement 49: 64-72, 1986.
18. Nord, C.E., Heimdahl, A., and Kager, L. The Impact of Different Antimicrobial Agents on the Normal Gastrointestinal Microflora of Humans. Reviews of Infectious Diseases, 6 (Supplement 1): S270-275, 1984.
19. Corpet, D.E. and Brugere, H.B. Antimicrobial Drug Residues in Foods: Evaluation of the No Effect on Human Microflora. Recent Res. Devel. In: *Antimicrob. Agents & Chemother.*, 1: 113-123, 1996.
20. Golberg, H.S., Goodman, R.N., Logue, J.T., and Handler, F.P. Long-Term, Low-Level Antibiotics and the Emergence of Antibiotic-Resistant Bacteria in Human Volunteers. In: *Antimicrobial Agents and Chemotherapy*, pp. 80-88, 1961.
21. Tancrede, C. and Barakat, R. Ecologial Impact of Low Doses of Oxytetracycline on Human Intestinal Microflora. J. Vet. Med. 42:35-39, 1987.
22. Corpet, D.E. Antibiotic Residues and Drug Resistance in Human Intestinal Microflora. Antimicrob. Agents Chemother. 31 (4): 587-593, 1987.
23. Brady, M.S., White, N., and Katz, S. Resistance Development Potential of Antibiotic/Antimicrobial Residue Levels Designated as "Safe Levels". Journal of Food Protection 56 (3): 229-233, 1993.
24. Egger, R. and Lebek, G. Selection of R-plasmid Harbouring *E. coli* by Subinhibitory Concentrations of Antibiotic. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. Orig. Reihe A 260: 361-368, 1985.
25. TechLab, Inc. Implementaion of an *In Vitro* System to Study the Effects of Low Doses of Antimicrobials on the Human Intestinal Flora. Year 5: Ciprofloxacin and the Barrier Effect. Final Report to the FDA-CVM. March 1, 2000 to August 31, 2000.
26. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of Low Doses of Antimicrobials on the Human Intestinal Flora. Year 4: Ciprofloxacin. Final Report to the FDA-CVM. September 11, 1998 to September 10, 1999.
27. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of Low Doses of Antimicrobials on the Human Intestinal Flora. 11th Quarterly Report to the FDA-CVM. March 11, 1998 to June 10, 1998.
28. Mc Conville, M.L., Dijkstra, J.W., Stamm, J.M., Van Saene, J.J.M., and Nouws, J.F.M. Effects of Sarafloxacin Hydrochloride on Human Enteric Bacteria Under Simulated Human Gut Conditions. The Veterinary Quarterly 17: 1-5, 1995.
29. Rumney, C.J., Coutts, J.T., Smith, J.S., and Rowland, I.R. Microbiological End-Point Determination of Antibiotics. Microbial Ecol. Health Dis. 10:3-11, 1998.
30. Corpet, D.E., Lumeau, S., and Corpet, F. Minimum Antibiotic Levels for Selecting a Resistance Plasmid in a Gnotobiotic Animal Model. Antimicrob. Agents Chemother. 33 (4): 535-540, 1989.
31. AFSSA. Effect of Tetracycline on the Bacterial Populations and the Metabolic Activity of the Human Intestinal Flora Implanted into Germ-free Mice: Treatment and Post-treatment Period. Fourth Quarterly Report 1997 to the FDA-CVM., October 3, 1997 to January 10, 1998.

32. AFSSA. Effect of Tetracycline on the Colonization Resistance of the Intestinal Human Flora Implanted into Germ-free Mice: Barrier Effect to a Pathogen Challenge. First Quarterly Report 1998 to the FDA-CVM. Laboratoire des Médicaments Vétérinaires, January 8, 1998 to April 20, 1998.
33. AFSSA. Effect of Neomycin on the Colonization Resistance of the Intestinal Human Flora Implanted into Germ-free Mice: Barrier Effect to a Pathogen Challenge. Second Quarterly Report 1998 to the FDA-CVM. Laboratoire des Médicaments Vétérinaires, April 22, 1998 to June 25, 1998.
34. AFSSA. Effect of Neomycin on the Bacterial Populations and Metabolic Activity of the Intestinal Human Flora Implanted into Germ-free Mice: Treatment and Post-treatment Periods. Fourth Quarterly Report 1998 to the FDA-CVM. Laboratoire des Médicaments Vétérinaires, October, 1998 to December, 1998.
35. AFSSA. Effect of Ciprofloxacin on the Bacterial Populations and Metabolic Activity of the Intestinal Human Flora Implanted into Germ-free Mice: Treatment and Post-treatment Periods. Fourth Quarterly Report 1999 to the FDA-CVM. Laboratoire des Médicaments Vétérinaires, March, 2000.
36. AFSSA. Effect of Ciprofloxacin on the Colonization Resistance of the Intestinal Human Flora Implanted into Germ-free Mice: Barrier Effect to a Pathogen Challenge. Second Quarterly Report 1999 to the FDA-CVM. Laboratoire des Médicaments Vétérinaires, May, 1999 to July, 1999.
37. Sande, M.A. and Mandell, G.L. Tetracycline, Chloramphenicol, Erythromycin, and Miscellaneous Antibacterial Agents. In: *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th edition, Pergamon Press, New York, pp. 1135-1137, 1990.
38. Manabe, Y.C. How to Diagnose *Clostridium difficile* diarrhea. Internal Medicine 18 (6): 29-41, 1997.
39. Vollaard, E.J., Clasener, H.A.L., and Janssen, A.J.H.M. The Contribution of *Escherichia coli* to Microbial Colonization Resistance. J. Antimicrob. Chemother. 26: 411-418, 1990.
40. Vollaard, E.J., Clasener, H.A.L., and Janssen, A.J.H.M. Co-trimoxazole Impairs Colonization Resistance in Healthy Volunteers. J. Antimicrob. Chemother. 30: 685-691, 1992.
41. Vollaard, E.J., Clasener, H.A.L., Janssen, A.J.H.M., and H.J.A. Wynne. Influence of Amoxicillin on Microbial Colonization Resistance in Healthy Volunteers. J. Antimicrob. Chemother. 25: 861-871, 1990.
42. Vollaard, E.J., Clasener, H.A.L., Janssen, A.J.H.M., and H.J.A. Wynne. Influence of Cefotaxime on Microbial Colonization Resistance in Healthy Volunteers. J. Antimicrob. Chemother. 26: 117-123, 1990.
43. Finegold, S.M. Interaction of Antimicrobial Therapy and Intestinal Flora. The American Journal of Clinical Nutrition 23 (11): 1466-1471, 1970.
44. Corpet, D.E. An Evaluation of Methods to Assess the Effect of Antimicrobial Residues on the Human Gut Flora. Veterinary Microbiology 35: 199-212, 1993.
45. Corpet, D.E. Microbiological Hazards for Humans of Antimicrobial Growth Promoter Use in Animal Production. Revue Med. Vet. 147 (12): 851-862, 1996.

46. Shlaes, D.M. Clinical Importance and Public Health Concerns for the Emergence of Antibiotic Resistant Organisms in the Human Intestinal Microflora. Veterinary and Human Toxicology 35 (Supplement 1): 24-27, 1993.
47. Rumney, C.J. and Rowland, I.R. *In Vivo* and *In Vitro* Models of the Human Colonic Flora. Critical Reviews in Food Science and Nutrition 31 (4): 299-331, 1992.
48. Carman, R.J., Vam Tassell, R.L., and Eilkins, T.D. Interactions Between Dietary Compounds and the Colonic Microflora. In: *Science for the Food Industry of the 21st Century, Biotechnology, Supercritical Fluids, Membranes and Other Advanced Technologies for Low Calorie, Healthy Food Alternatives.* Yalpani, M. (ed.). ATL Press, pp. 314-342, 1993.
49. Hoverstad, T., Carlstedt-Duke, B., Lingaas, E., Norin, E., Saxerholt, H., Steinbakk, M. and Midtvedt, T. Influence of Oral Intake of Seven Different Antibiotics on Faecal Short-Chain Fatty Acid Excretion in Healthy Subjects. Scand. J. Gastroenterol. 21:997-1003, 1986.
50. Corpet, D.E. Current Status of Model for Testing Antibiotic Residues. Veterinary and Human Toxicology 35 (Supplement 1): 37-46, 1993.
51. Boisseau, J. Basis for the Evaluation of the Microbiological Risks due to Veterinary Drug Residues in Food. Veterinary Microbiology 35: 187-192, 1993.
52. Woodward, K.N. The Use of Microbiological End-Points in the Safety Evaluation and Elaboration of Maximum Residue Limits for Veterinary Drugs Intended for Use in Food Producing Animals. J. Vet. Pharmacol. Therap. 21:47-53, 1998.
53. Cerniglia, C.E. and Kotarski, S. Evaluation of Veterinary Drug Residues in Food and Their Potential to Affect Human Intestinal Microflora. Regulatory Toxicology and Pharmacology 29: 238-261, 1999.
54. Kotarski, S.F. and Boucher, J.F. Variability Encountered in Model Systems Designed to Test Antimicrobial Effects on the Intestinal Microflora. Microbial Ecology in Health and Disease, Supplement 1, 2000.
55. Nouws, J.F.M., Kuiper, H., Van Klingeren, B., and Kruyswijk, P.G. Establishment of a Microbiologically Acceptable Daily Intake of Antimicrobial Drug Residues. Veterinary Quarterly 16 (3): 152-156, 1994.
56. Mc Conville, M.L., Dijkstra, J.W., Stamm, J.M., Van Saene, J.J.M., and Nouws, J.F.M. 1995. Effects of Sarafloxacin Hydrochloride on Human Enteric Bacteria Under Simulated Human Gut Conditions. The Veterinary Quarterly 17: 1-5.
57. Silley, P. Personal communication. A Simple *In Vitro* Gut Model. Workshop "Microbiological Safety of Drug Residues in Food". Rockville, MD, September 20-21, 2000.
58. Miller, T.L. and Wolin, M.J. Fermentation by the Human Large Intestine Microbial Community in an *In Vitro* Semicontinuous Culture System. Applied and Environmental Microbiology 42 (3): 400-407, 1981.
59. Manning, B.W., Federle, T.W., and Cerniglia, C.E. Use of a Semicontinuous Culture System as a Model for Determining the Role of Human Intestinal Microflora in the Metabolism of Xenobiotics. Journal of Microbiological Methods 6: 81-94, 1987.
60. Campbell, W.L., Franklin, W., and Cerniglia, C.E. Validation Studies on an *In Vitro* Semicontinuous Culture System Designed to Simulate a Bacterial Ecosystem of the Human Intestine. Journal of Microbiological Methods, 16: 239-252, 1992.

61. Ushijima, T. and Ozaki, Y. Potent Antagonism of *Escherichia coli, Bacteroides ovatus, Fusobacterium varium*, and *Enterococcus faecalis*, Alone or in Combination, for Enteropathogens in Anaerobic Continuous Flow Cultures. Med. Microbiol. 22: 157-163, 1986.
62. Edwards, C.A., Duerde, B.I., and Read, N.W. Metabolism of Mixed Human Colonic Bacteria in a Continuous Culture Mimicking the Human Cecal Contents. Gastroenterology 88 (6): 1903-1909, 1985.
63. Gibson, G.R., Cummings, J.H., and MacFarlane, G.T. Use of a Three-Stage Continuous Culture System to Study the Effect of Mucin on Dissimilatory Sulfate Reduction and Methanogenesis by Mixed Populations of Human Gut Bacteria. Applied and Environmental Microbiology 54(11): 2750-2755, 1988.
64. Lebek, G. and Egger, R. The Effect of Low Levels of Antibiotics on the Selection of Resistance in Intestinal Bacteria. *In Vitro* Observations. 1983.
65. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. First Quarterly Report, 11 September to 10 December, 1995.
66. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. Second Quarterly Report, 11 December, 1995 to 10 March, 1996.
67. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. Third Quarterly Report, 1996.
68. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. First Annual Report, 11 September, 1995 to 10 September, 1996.
69. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. Fifth Quarterly Report, 11 September, 1996 to 10 December, 1996.
70. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. Sixth Quarterly Report, 11 December, 1996 to 10 March, 1997.
71. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. Seventh Quarterly Report, 11 March, 1997 to 10 June, 1997.
72. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. Eighth Quarterly Report, 11 June, 1997 to 10 September, 1997.
73. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. Ninth Quarterly Report, 11 September, 1997 to 10 December, 1997.
74. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. Tenth Quarterly Report, 11 December, 1997 to 10 March, 1998.
75. TechLab, Inc. Implementation of an *In Vitro* System to Study the Effects of low Doses of Antimicrobials on the Human Intestinal Flora. Twelfth Quarterly Report, 11 June, 1998 to 10 September, 1998.

76. Rollins, L.D., Gaines, S.A., Pocurull, D.W., and Mercer, H.D. Animal Model for Determining the No-Effect Level of an Antimicrobial Drug on Drug Resistance in the Lactose-Fermenting Enteric Flora. Antimicrob. Agents Chemother. 7(5): 661-665, 1975.
77. Gaines, S.A., Rollins, L.D., Silver, R.P., and Washington, M. Effect of Low Concentrations of Dihydrostreptomycin on Drug Resistance in Enteric Bacteria. Antimicrob. Agents Chemother. 14(2): 252-256, 1978.
78. Gaines, S.A., Rollins, L.D., Williams, R.D., and Selwyn, M. Effect of Penicillin and Virginiamycin on Drug Resistance in Lactose-Fermenting Enteric Bacteria. Antimicrob. Agents Chemother. 17(3): 428-433, 1980.
79. Corpet, D.E. and Lumeau, S. Antibiotic Residues and R-Plasmid Selection: Are *In Vitro* Methods Good Models? Zbl. Bakt. Hyg. A 264: 178-184, 1987.
80. Corpet, D.E. The Effect of Bambermycin, Carbadox, Chlortetracycline, and Olaquindox on Antibiotic Resistance in Intestinal Coliforms: A New Animal Model. Ann. Microbiol. 135 A: 329-339, 1984.
81. Hazenberg, M.P., Bakker, M., and Verschoor-Burggraaf, A. Effects of the Human Intestinal Flora on Germ-free Mice. J. Appl. Bacteriol. 50: 95-106, 1981.
82. Andremont, A., Raibaud, P. and Tancrede, C. Effect of Erythromycin on Microbial Antagonism: A Study in Gnotobiotic Mice Associated with a Human Fecal Flora. J. Infect. Dis. 148(3): 579-587, 1983.
83. Leonard, F., Andremont, A., and Tancrede, C. *In Vivo* Activity of Nifurzide and Nifuroxazide in Intestinal Bacteria in Man and Gnotobiotic Mice. J. Appl. Bacteriol. 58: 545-553, 1985.
84. Pecquet, S., Andremont, A., and Tancrede, C. Selective Antimicrobial Modulation of the Intestinal Tract by Norfloxacin in Human Volunteers and in Gnotobiotic Mice Associated with a Human Fecal Flora. Antimicrob. Agents Chemother. 29(6): 1047-1052, 1986.
85. Wong, W.C., Hentges, D.J., and Dougherty, S.H. Adequacy of the Human Faecal Microbiota Associated Mouse as a Model for Studying the Ecology of the Human Intestinal Tract. Microbial Ecology in Health and Disease 9: 187-198, 1996.
86. Massey, R.C., Key, P.E., Mallet, A.K., and Rowland I.R. An Investigation of the Endogenous Formation of Apparent Total N-Nitroso Compounds in Conventional Microflora and Grem-Free Rats. Fd. Chem. Toxic. 26(7): 595-600, 1988.
87. AFSSA. Effect of Tetracycline on the Bacterial Populations and the Metabolic Activity of the Human Intestinal Flora Implanted into Germ-Free Mice. Summary Report 1997 to the FDA-CVM. Laboratoire des Médicaments Vétérinaires, 1997.
88. Guyomard, A. Human Flora Associated (HFA) Mice: Effect of Selected Antimicrobials on the Human Intestinal Microflora. Microbial Ecology in Health and Disease (submitted for publication).
89. WHO. Evaluation of Certain Veterinary Drug Residues in Food. In: *Thirty-second Report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series 763*. World Health Organization, Geneva, Switzerland, pp. 1-17, 1988.
90. WHO. Evaluation of Certain Veterinary Drug Residues in Food. In: *Thirty-sixth Report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series 799*. World Health Organization, Geneva, Switzerland, pp. 7-14; 41-44, 1990.

91. WHO. Evaluation of Certain Veterinary Drug Residues in Food. In: *Thirty-eighth Report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series 815*. World Health Organization, Geneva, Switzerland, pp. 32-33, 1991.
92. WHO. Evaluation of Certain Veterinary Drug Residues in Food. In: *Forty-second Report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series 851*. World Health Organization, Geneva, Switzerland, pp. 4-5; 23-24, 1995.
93. WHO. Evaluation of Certain Veterinary Drug Residues in Food. In: *Forty-fifth Report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series 864*. World Health Organization, Geneva, Switzerland, pp. 3; 26-28, 1996.
94. WHO. Evaluation of Certain Veterinary Drug Residues in Food. In: *Forty-seventh Report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series 876*. World Health Organization, Geneva, Switzerland, pp. 1-5, 1998.
95. WHO. Evaluation of Certain Veterinary Drug Residues in Food. In: *Fiftieth Report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series 888*. World Health Organization, Geneva, Switzerland, pp. 21-54, 1999.
96. Chamberlain, P.L. Historical Perspective on the Regulation of Antimicrobial Residues in Food. Microbial Ecology in Health and Disease, Supplement 1, 2000.
97. Freischem, B. J. Regulation of Residues of Antimicrobial Veterinary Medicinal Products in Food in the European Union. Microbial Ecology in Health and Disease, Supplement 1, 2000.